Targeted degradation of mRNA in *Xenopus* oocytes and embryos directed by modified oligonucleotides: studies of An2 and cyclin in embryogenesis

John M.Dagle[1], Joseph A.Walder[1,2] and Daniel L.Weeks[1]*
[1]Department of Biochemistry, University of Iowa, Iowa City, IA 52242 and [2]Integrated DNA Technologies, Inc., Coralville, IA 52241, USA

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ABSTRACT

We have designed antisense oligodeoxyribonucleotides which are both highly resistant to nucleolytic degradation and also serve as substrates for ribonuclease H. Using these compounds we have targeted the specific degradation of several maternal mRNAs present in Xenopus laevis oocytes and early embryos. Several internucleoside linkages at both the 3′ and 5′ ends of the oligonucleotides were modified as phosphoramidates to provide complete protection against exonucleases, the predominant nucleolytic activity found in both oocytes and embryos. Eight internal linkages were left unmodified to provide a substrate for RNase H. Degradation of specific embryonic mRNAs was accomplished using subtoxic amounts of the modified oligonucleotides. Specific depletion of An2, a localized mRNA encoding the alpha subunit of the mitochondrial ATPase, produced embryos that gastrulated later than control embryos and arrested in development prior to neurulation. A modified oligonucleotide targeting Xenopus cyclin B1 and cyclin B2 mRNA was also synthesized. Following the injection of one blastomere of a two-cell embryo with the anti-cyclin oligonucleotide, cell division in that half of the embryo was inhibited, demonstrating the in vivo importance of these cyclins in mitosis. The oligonucleotide analogs described here should be useful in studying developmentally significant proteins in Xenopus.

INTRODUCTION

Antisense oligodeoxyribonucleotides (ODNs) have been used successfully to specifically inhibit gene expression in a variety of eukaryotic systems (for review, see 1–3). Several possible mechanisms for antisense inhibition in eukaryotic cells exist. ODNs can bind to complementary sequences in DNA, thereby forming a triplex structure which may interfere directly with transcription (4). Alternatively, antisense ODNs may act posttranscriptionally. Upon hybridization of an antisense ODN to its complementary mRNA, a stable hybrid is formed, the fate of which depends upon the nature of the ODN. ODNs with unmodified internucleoside linkages form heteroduplexes with RNA that are substrates for cellular ribonuclease H (RNase H). Phosphorothioate-modified ODNs can also direct the degradation of complementary mRNA by RNase H (5). RNase H will cleave the targeted mRNA at the site of hybridization to the ODN. Following this initial cleavage, other endogenous ribonuclease activities may degrade the remainder of the mRNA. After directing the cleavage of one mRNA, the intact ODN can dissociate and rehybridize to additional messages. Therefore, the potential exists for substoichiometric amounts of ODN to mediate the complete degradation of a specific mRNA pool. Antisense studies in reticulocyte lysates have shown that ODN-mediated cleavage of a targeted message by RNase H is the most effective means of antisense inhibition of translation (6).

ODNs that are completely modified with neutral alkylphosphonate or phosphoramidate internucleoside linkages do not form substrates for RNase H (7,8). As a result, high concentrations of these compounds are required for effective steric inhibition of translation. Oligonucleoside methylphosphonates, for example, typically require concentrations of 100 μM or greater for significant inhibition of translation in vitro (7,9). Unmodified ODNs in the same systems are effective in the range of 1–5 μM (10,11). The major advantage of the nonionic ODNs is their resistance to nuclease activities. In contrast to unmodified ODNs, which are degraded by cellular nucleases (12–15), the nonionic phosphate-modified compounds are extremely resistant to both exonucleases and endonucleases (16,17).

Our interest in the role of localized maternal mRNAs in Xenopus development led us to investigate the utility of antisense ODNs in exploring events of early embryogenesis. This approach seemed attractive since the presence of RNase H activity in Xenopus oocytes had been previously established (18,19). Early development in Xenopus relies on the pool of proteins and mRNAs present in the egg prior to fertilization. Because embryos are transcriptionally inactive until the 4000-cell stage (20,21), removal of a specific mRNA present in the maternal pool by the action of antisense ODNs will irrevocably eliminate synthesis of the protein it encodes during early development. Several laboratories have demonstrated the potential of using antisense

* To whom correspondence should be addressed

nucleic acids to inhibit specific mRNA utilization in Xenopus oocytes (15,19,22−27). Attempts to utilize antisense RNA and ODNs during embryogenesis, however, have not been successful. Three obstacles hinder the usefulness of antisense ODNs for the study of maternal mRNA function in Xenopus embryos. First, unmodified ODNs are susceptible to nuclease degradation. Secondly, modifications that increase stability often decrease the capacity of the ODN to serve as an RNase H substrate. Finally, the amount of unmodified ODN needed to effectively inhibit translation carries with it nonspecific toxicity (15, for review, see 1).

In the present work, we have incorporated the favorable properties of both modified and unmodified ODNs in the design of antisense compounds. We have modified several of the internucleoside linkages at both the 3′ and 5′ termini with phosphoramidates to endow the ODN with exonuclease- and partial endonuclease-resistance, while retaining a number of consecutive internal phosphodiesters to take advantage of the endogenous RNase H activity in Xenopus oocytes and embryos. Using these ODN analogs we have investigated the roles of An2 and cyclin mRNAs in early embryogenesis. Inhibiting the translation of An2, a localized message which encodes the alpha subunit of mitochondrial ATPase (28), resulted in the arrest of development shortly after gastrulation. Preventing synthesis of cyclin B1 and cyclin B2, which are components of Xenopus maturation-promoting factor (29), inhibited embryonic cell division, demonstrating, in vivo, an essential role of the cyclins in the cell cycle.

EXPERIMENTAL PROCEDURES

Materials

2-Methoxyethylamine was purchased from Aldrich and stored over 3-Å molecular sieves. ODN synthesis reagents were from Applied Biosystems. Human chorionic gonadotropin, dithiothreitol and Ficoll-400 were purchased from Sigma. Proteinase K was obtained from Beckman. SP6 polymerase and nucleotide triphosphates were purchased from Promega. All radioactive nucleotides were purchased from Amersham. RNase A and RNase T1 were from Boehringer. Xenopus laevis were purchased from Xenopus I (Ann Arbor, MI).

Synthetic ODNs

ODNs were made on an ABI 381A DNA synthesizer using hydrogen phosphonate chemistry (30). Coupling, oxidation and sulfurization were performed according to the manufacturer's recommendations. Oxidative amidation was performed manually by treating hydrogen phosphonate diesters with 3 ml of 10% methoxyethylamine in carbon tetrachloride for one hr (31). The partially modified ODNs were synthesized in three blocks. After the desired number of 3′ residues were condensed, the amidation reaction was carried out. Next, the central region was synthesized and subsequently oxidized with 5% I_2 in tetrahyrofuran:pryidine:H_2O (90:5:5) according to the manufacturer. Phosphorothioate linkages were prepared by sulfurization of the central stretch with 5% S_8 in CS_2:pyridine:TEA (48:48:2) for between 2 and 4 hr. Finally the 5′ portion of the ODN was synthesized and amidated as before. The ODN was removed from the solid support with concentrated NH_4OH at 60 °C for 7 hr. The DMT-protected ODNs were purified by reversed-phase HPLC (6), detritylated in 80% acetic acid for 30 min and again purified by reversed-phase HPLC. The ODNs were then passed through a NAP-5 gel filtration column (Pharmacia) twice, dried under vacuum and resuspended in sterile water.

ODN labeling

The ODNs utilized to study degradation were internally labeled at the eighth internucleoside linkage from the 5′ end. 5′ATGACTGC was labeled at the 5′ terminus with gamma ^{32}P ATP and T4 polynucleotide kinase. The labeled 3′ fragment was then ligated at room temperature for 6 hr to CTGACAAC in the presence of a complementary 21-mer using T4 DNA ligase. The final product, a 16-mer, was purified by electrophoresis on a 20% polyacrylamide-7M urea gel. The ODN was eluted from the gel, applied to a NENsorb column (Du Pont), eluted with 50% 1-propanol, dried in a SpeedVac Concentrator (Savant) and resuspended in water. The labeled modified ODN was prepared similarly. The 3′ fragment utilized was ATGACT*G*C, where * denotes a methoxyethylphosphoramidate internucleoside linkage. It was ligated to the 5′ fragment, C*T*G*ACAAC, as described above to yield C*T*G*ACAACATGACT*G*C.

Microinjection of oocytes and embryos

Stage 6 oocytes (32) were obtained from mature frogs and maintained in 1× MBSH (33). Eggs were obtained and fertilized as previously described (34). ODNs were injected into the cytoplasm of oocytes as described by Colman (33). Fertilized eggs were dejellied for 10−15 min in either 5 mM DTT, 50 mM HEPES pH 7.5 or in 2% cysteine, 0.1× MBSH. The eggs were then rinsed and placed in 0.1× MBSH containing 5% Ficoll-400 prior to injection.

ODN degradation

At various times following injection of labeled ODN, the oocytes or embryos were frozen in dry ice and thawed in 200 μl phenol with vigorous vortexing. The solution was extracted once with water. Following the addition of 200 μl chloroform and 400 μl 0.2% SDS, the phenol layer was extracted with water twice more. Aqueous fractions were pooled, extracted once with chloroform and dried in a SpeedVac Concentrator. The residue was resuspended in water and the amount of radioactivity was determined. Greater than 90% of the radioactivity was extracted for each sample. Equal amounts of radioactivity were then analyzed by electrophoresis using a 20% polyacrylamide-7M urea gel which was subsequently exposed at −70 °C to Kodak X-OMAT AR film with an intensifying screen.

RNA probes

The antisense An2 RNA probe used was an SP6 transcript made from BglI-cut or EcoRV-cut pSP72An2.1P, a plasmid constructed by removing a PstI fragment from pSP65An2.1 (28). Antisense histone H4 probes were generated as previously described (34). Transcription reactions with SP6 RNA polymerase were performed as described earlier (35). All probes were purified on a 6% polyacrylamide-7M urea gel prior to use.

Analysis of RNA

Oocyte and embryo RNA was extracted as previously described (36), and quantitated by RNase protection. Briefly, defined oocyte or embryo equivalents of total RNA were precipitated with the specific antisense probe. The RNA was resuspended in 30 μl 80% formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA. The solution was heated to 85°C for 5 min, then incubated at least 10 hr at 45°C. Following this incubation, 350

μl of 10 mM Tris, pH 7.4, 300 mM NaCl, 5 mM EDTA containing 20 U/ml bovine pancreatic RNase and 100 U/ml RNase T1 were added. After 1 hr at room temperature, 10 μl 20% SDS and 2.5 μl 20 mg/ml proteinase K were added. Following a 20 min incubation at 37°C the solution was extracted with 400 μl phenol:chloroform (50:50). The aqueous layer was precipitated and resuspended in 6 μl 5 M urea, 0.1% sarkosyl, 0.5 mM EDTA. The samples were heated for 5 min at 85°C, and loaded onto a 6% polyacrylamide-7M urea gel. The gel was fixed, dried and exposed at −70°C to Kodak X-OMAT AR film using an intensifying screen. Quantitation of radioactivity present in gels was performed using a radioisotope imaging system (Ambis).

A

Anti An2-16

An2-NON 5' C*T*G*A C A A C A T G A*C*T*G*C

An2-NSN 5' C*T*G*A*C^A^A^C^A^T^G^A*C*T*G*C

Anti CAT-16

CAT-NON 5' T*G*G*T A T A T C C A G *T*G*A*T

CAT-NSN 5' T*G*G*T^A^T^A^T^C^C^A^G *T*G*A*T

B

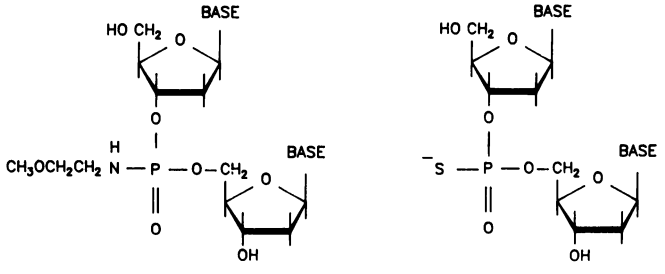

Figure 1. Antisense ODNs and Phosphodiester Analogs. (A) The nucleotide sequence of the anti-An2 ODNs corresponds to the region of the An2 mRNA containing the initiation codon. The anti-CAT ODNs are complementary to a sequence in the coding region of the chloramphenicol acetyltransferase mRNA. Methoxyethylphosphoramidate linkages are designated as *. Phosphorothioate bonds are represented as ^. (B) Dinucleotides containing a methoxyethylphosphoramidate (left) and a phosphorothioate (right) linkage.

RESULTS

ODN modifications increase stability in embryos and oocytes

The ODNs chosen for this study were designed to be nuclease resistant, yet capable of forming heteroduplexes that are substrates for RNase H. The ODNs synthesized are shown in Figure 1A. Methoxyethylphosphoramidate and phosphorothioate internucleoside linkages are illustrated in Figure 1B. The sequences are complementary to the translation initiation region of the Xenopus An2 mRNA, or to the coding region of the bacterial chloramphenicol acetyltransferase (CAT) mRNA. ODN An2-NON was a 16-mer possessing three phosphoramidate linkages at the 5′ terminus and four modified linkages at the 3′ terminus. An2-NSN was identical to An2-NON, except that its central anionic region was composed of phosphorothioates instead of unmodified phosphodiesters. CAT-NON and CAT-NSN were modified exactly as the An2 series. Stability studies were carried out on the unmodified An2 ODN and on a derivative of An2-NON containing 3 phosphoramidate linkages at the 5′ end of the molecule and only 2 at the 3′ terminus ($N_3O_{10}N_2$). Radiolabeled An2-NON ($N_3O_8N_4$) could not be constructed using T4 DNA ligase due to limitations in the activity of the enzyme with highly modified DNA substrates. Each labeled 16-mer carried the radioactive phosphorous at the eighth internucleoside linkage from the 5′ terminus. This labeling strategy is preferable to the use of 5′-end-labeled ODNs as it eliminates possible confusion due to the presence of phosphatases.

The rate of degradation of ODNs was investigated by injecting into oocytes a solution containing both the unmodified and phosphoramidate-modified ($N_3O_{10}N_2$) An2 ODNs. (Fig. 2A). Lane C demonstrates the uninjected ODN solution. The unmodified ODN (U) was rapidly degraded following injection into oocytes. Some degradation was already evident in the few seconds required for the injection and immediate processing of the oocytes (lane 0 min). After 10 min, the unmodified ODN

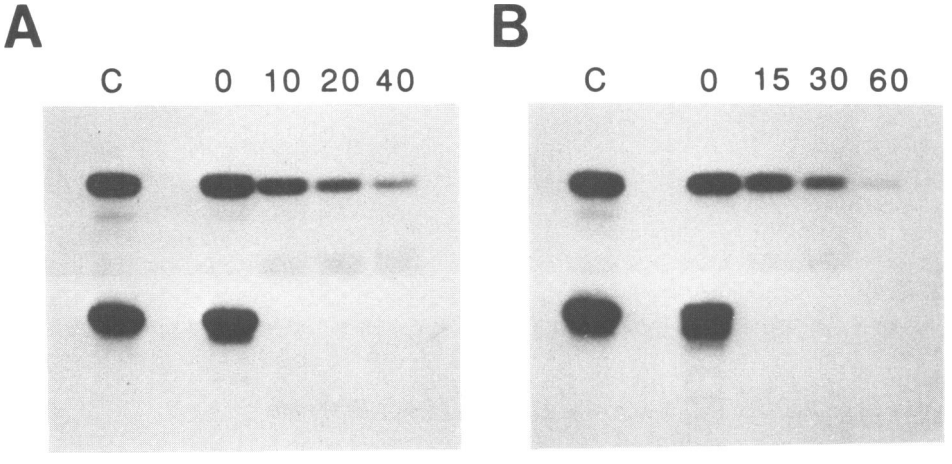

Figure 2. Degradation of Unmodified and Phosphoramidate-modified ODNs in Xenopus Oocytes and Embryos. (A) A mix of labeled modified (15 ng) and unmodified (15 ng) ODNs were injected into oocytes. Immediately after injection (0) and at 10, 20, and 40 min post-injection oocytes were frozen, lysed in phenol, and ODNs were extracted. Equal amounts of radioactivity were analyzed by electrophoresis on a 20% polyacrylamide-7M urea gel. An uninjected sample containing both modified (M) and unmodified (U) ODNs is shown in lane C. (B) Injection of the ODN mix into embryos. Embryos were treated as in (A), except were harvested at 0, 15, 30 and 60 min post-injection.

was nearly completely degraded. The modified ODN (M) was degraded far more slowly and was present at detectable levels 40 min after injection. The major degradation product of the unmodified 16-mer observed initially (lane 0) was a 15-mer. This product must have resulted from cleavage by either a 5′ or a 3′ exonuclease, or a combination of both activities. Additionally, an extended exposure of Figure 2A revealed, in lane 0, a ladder of degradation products below the unmodified ODN differing by a single nucleotide (data not shown). The involvement of an exonuclease was further supported by the ability to increase ODN stability by modifying the termini.

Degradation of the modified ODN by ooctyes established the existence of an endonucleolytic activity. In contrast to a ladder of degradation products, hydrolysis of the modified ODN occurred internally with the production of metastable fragments which migrated electrophoretically as a doublet approximately midway between the modified and unmodified bands.

The degradation of the unmodified ODN appeared to be even more rapid in embryos (Fig. 2B). Less than one min after injection, approximately half of the unmodified ODN was degraded from a 16-mer to a 15-mer (lane 0), and within 15 min full length ODN was almost undetectable. In embryos, initial degradation of the unmodified ODN to the 15-mer again indicates an exonucleolytic pathway which could be blocked by terminal phosphoramidate modifications. At 15 min after injection into embryos, about half of the modified ODN was intact. Degradation occurred by an endonucleolytic activity similar to that seen in oocytes, with the production of the same principle cleavage fragments.

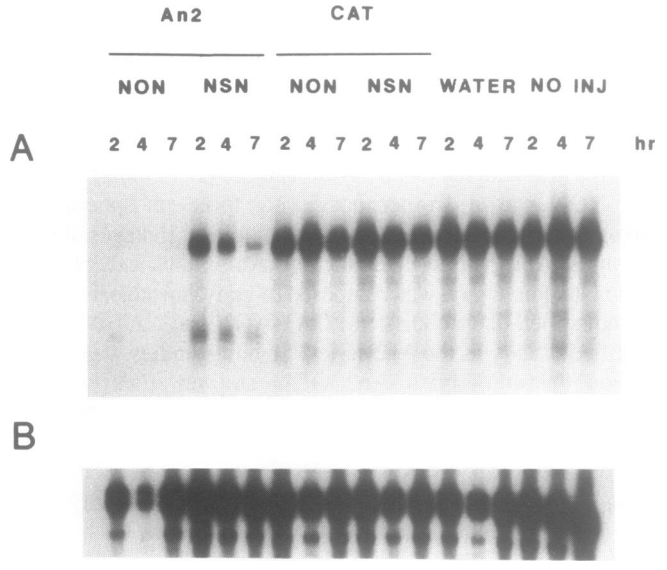

Figure 3. Degradation of An2 mRNA in Oocytes Following Injection of An2-NON and An2-NSN. Stage VI oocytes were injected with 50 ng of the indicated ODN and harvested at 2, 4 and 7 hr. RNA was isolated and quantitated by RNase protection using a labeled antisense An2 probe (A) or histone H4 probe (B). An2 analysis was performed using RNA from 2.25 oocytes and histone analysis using the RNA from 0.45 oocytes per lane. Protected fragments were electrophoresed on a 6% denaturing polyacrylamide gel and visualized by autoradiography.

Specific degradation of An2 mRNA in oocytes and embryos

To examine the effect of antisense ODNs on An2 mRNA levels, we injected 50 ng of the ODNs shown in Figure 1A into the cytoplasm of stage VI oocytes (32). At 2, 4 and 7 hr post-injection, RNA was isolated and the amounts of An2 and histone H4 mRNA were quantitated by RNase protection (35) (Fig. 3). Injection of An2-NON resulted in nearly complete degradation of An2 mRNA within 2 hr (Fig. 3A). The An2-NSN ODN also directed specific degradation of An2 mRNA in oocytes, but at a much slower rate than An2-NON. There was continuous degradation of An2 message throughout the 7 hr An2-NSN time course, indicating the presence of intact ODN in the oocyte

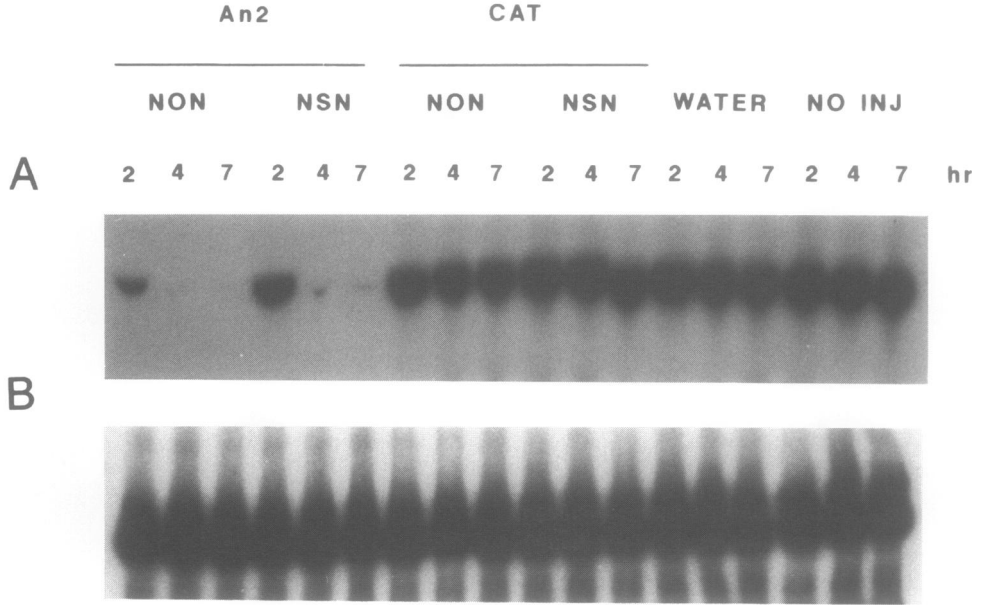

Figure 4. Degradation of An2 mRNA in Embryos Following Injection of AN2-NON and An2-NSN. Embryos just prior to first cleavage were injected with 50 ng of the indicated ODN and harvested at 2, 4 and 7 hr. RNA was isolated and quantitated by RNase protection using a labeled antisense An2 probe (A) or histone H4 probe (B). An2 analysis was performed using RNA from 2 embryos and histone analysis using the RNA from 0.6 embryos. Protected bands were electrophoresed on a 6% denaturing polyacrylamide gel and visualized by autoradiography.

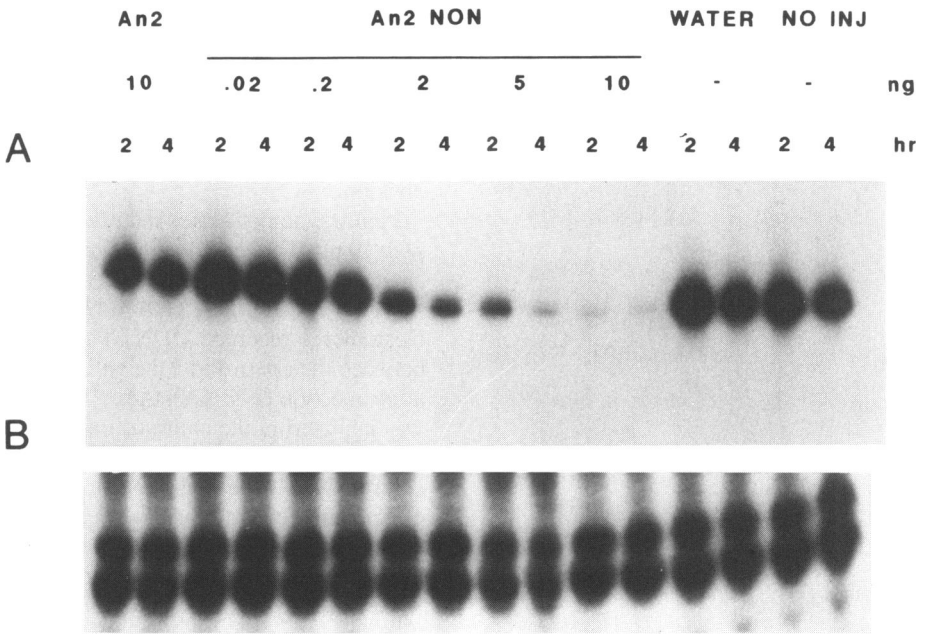

Figure 5. Effect of An2-NON Concentration on the Degradation of An2 mRNA in Embryos. Embryos just prior to first cleavage were injected with the indicated amounts of An2-NON and harvested at 2 and 4 hr. RNA was isolated and quantitated by RNase protection using a labeled antisense An2 probe (A) or histone H4 probe (B). An2 analysis was performed using RNA from 2.25 embryos and histone analysis using the RNA from 0.45 embryos. Protected bands were electrophoresed on a 6% polyacrylamide-7M urea gel and visualized by autoradiography.

several hours following injection. In the An2-NSN lanes, a band of lower intensity corresponding to the 3′ cleavage fragment can be seen below the fully protected band. This fragment is also faintly visible following injection of An2-NON. Injection with either CAT-NON or CAT-NSN had no effect on An2 message levels compared to uninjected and water injected controls. The histone H4 controls (Fig. 3B) established that approximately equal amounts of RNA were assayed in each lane, with the exception of the AN2-NON 4 hr time point, and that degradation of An2 mRNA is sequence specific.

In an attempt to attenuate An2 mRNA levels in developing embryos, 50 ng of each ODN shown in Figure 1A were injected into fertilized eggs just prior to first cleavage. Figure 4A demonstrates the reduction in the level of An2 message following injection with An2-NON. Greater than 90% of An2 mRNA had been degraded by 2 hr, and by 4 hr the message was undetectable. As was seen in oocytes, ODN An2-NSN had little effect 2 hr after injection. In contrast, however, intact An2 mRNA was nearly undetectable after 4 hr. Injection of CAT-NON, CAT-NSN, or water yielded levels of An2 mRNA comparable to uninjected control embryos. Levels of histone H4 message were not influenced by injection of water or any ODN (Fig. 4B). The presence of An2 message 2 hr after injection with either An2-NON or An2-NSN, and its subsequent disappearance 2 hr later, indicated that antisense ODNs were present in the embryos for several hours. In both oocytes and embryos, the antisense activity of An2-NON was greater than that of An2-NSN. For that reason, and because of reports of nonspecific, hybridization-independent, biological effects of phosphorothioate ODNs (37), subsequent studies were performed using An2-NON.

The extent of reduction of An2 message by An2-NON is dose-dependent

To determine the amount of An2-NON required to completely degrade the An2 message, fertilized eggs were injected, just prior to first cleavage, with either 10 ng of unmodified An2 ODN or from 0.02 to 10 ng of An2-NON. RNA was isolated 2 and 4 hr after injection and levels of An2 and histone H4 mRNA were determined by RNase protection. The extent of An2 message degradation increased with increasing amounts of injected ODN (Fig. 5A). Histone H4 mRNA levels, however, were unchanged over the range of An2-NON concentrations tested (Fig. 5B), demonstrating the specificity of the antisense effect. Results for An2 mRNA are summarized in Table 1. Injection of 10 ng of unmodified ODN was needed to reduce An2 mRNA levels by about 50% in 4 hr. This same attenuation could be achieved with approximately 0.5 ng of An2-NON, indicating that the modified ODNs are 20 fold more active antisense compounds. Injection of 5 ng of An2-NON resulted in the degradation of 96% of the An2 mRNA by 4 hr. The 50−100 ng of unmodified ODN necessary to reduce An2 levels by 95% would produce severe toxic effects (15). The increased level of antisense activity seen with the modified ODNs roughly corresponds to their increased nuclease resistance.

Table 1. Oligonucleotide-directed Degradation of An2 mRNA

Injection	Amount ng	Relative An2 Level 2 hr	4 hr
No injection	–	100	70
Water	–	100	77
An2 Unmodified	10	61	54
An2-NON	0.02	96	83
An2-NON	0.2	81	64
An2-NON	2	31	18
An2-NON	5	17	4
An2-NON	10	2	2

Radioactivity in each lane of Figure 5 was quantitated and compared to the 2 hour, no injection control, which was arbitrarily assigned a value of 100.

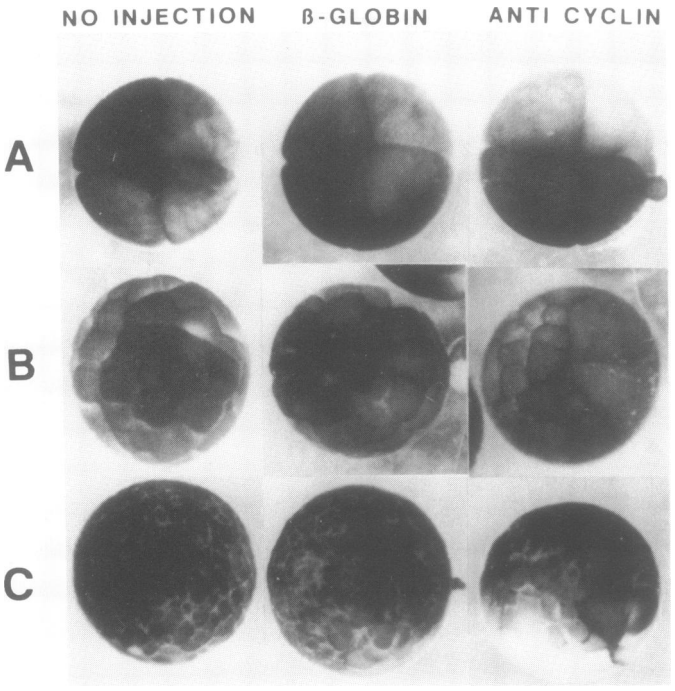

Figure 6. Inhibition of Cell Division in Xenopus Embryos by the Injection of an ODN Targeted Against Cyclin B1 and B2. Following first cleavage, one blastomere of a 2-cell embryo was injected with 10 ng of either a modified β-globin ODN or an ODN complementary to a sequence common to both cyclin B1 and cyclin B2 mRNA. The embryos were photographed at 20 (A), 100 (B), and 200 (C) min after injection. The photographs are positioned so the site of injection faces right.

Morphologic changes seen in embryos injected with antisense ODNs to An2 mRNA

There have been several reports of non-specific toxicity associated with the injection of high concentrations of ODNs into embryos (1,15,27). In our hands, injection of 50 ng of any ODN resulted in retarded early cleavages and death of the embryo prior to gastrulation. Because the modified analogs were more effective antisense agents than the unmodified ODNs, we were able to reduce the amount of material injected by 10−20 fold and still degrade >95% of the targeted mRNA. First cleavage embryos injected with 5 ng of a control modified ODN (either CAT-NON or a sense murine β-globin ODN, 5′A*T*G*GTGCACCT-G*A*C*T where * indicates a phosphoramidate linkage) developed into normal swimming tadpoles at the same frequency as those injected with water (about 30%). The injection of 2 ng of An2-NON resulted in degradation of 80% of An2 mRNA and consequently a 2−3 hr delay in the onset of gastrulation. In contrast to the toxicity observed following the introduction of high levels of any ODN, An2-NON-injected embryos proceeded through gastrulation, but development stopped shortly after stage 11 (38), a late gastrula stage. No embryos injected with 2 ng of An2-NON proceeded to hatch. When 10 fold less AN2-NON was injected, resulting in only 19% depletion of the maternal pool of An2 mRNA (Fig. 5A), there was no significant difference in survival between oligo-injected and water-injected embryos.

An ODN directed against Xenopus cyclin mRNA inhibits cell division

ODNs have recently been used in activated Xenopus egg extracts to examine the importance of cyclin B1 and B2 in early cell division (39). For embryonic cells to enter mitosis, cyclin protein must be synthesized from maternal stores of cyclin mRNA. Subsequent degradation of the protein is necessary for cells to exit from mitosis (40,41). New cyclin protein is alternately synthesized and degraded with each cell cycle. Inhibition of cyclin expression should prevent mitosis and, therefore, subsequent cell division.

Figure 6 demonstrates the effect of injecting stage 2 embryos with 10 ng of either a murine β-globin ODN or an ODN targeted against a region common to both cyclin B1 and cyclin B2 (G*T*A*C*ATCTCTTCA*T*A*T*T). Only one of the two blastomeres received ODN. There was no obvious difference between the control and the anti-cyclin treated embryos 20 min after injection (Fig. 6A). After 100 min, however, cell division was inhibited in the embryos injected with the anti-cyclin ODN (Fig. 6B), while uninjected embryos and those injected with control ODN appeared normal. At 200 min after injection the effect was even more pronounced (Fig. 6C). The uninjected and control embryos continued normal development, while the anti-cyclin injected embryos contained abnormally large cells resulting from an early block in cell division. In two separate studies, all embryos injected with the anti-cyclin ODN exhibited a block in cell division on the injected side, while normal development continued for several hours on the uninjected side. No embryo injected with the β-globin ODN displayed such a morphology.

DISCUSSION

Degradation of ODNs in Xenopus oocytes and embryos

The ability of antisense ODNs to direct mRNA degradation and consequently prevent the synthesis of a specific protein is dependent upon the stability of the ODN. Regardless of the ODN's site of action, nucleolytic degradation of the molecule will ultimately attenuate the antisense effect. We have examined nucleolytic activities in Xenopus oocytes and embryos to determine the pathways of ODN degradation and assess the effect of phosphate modifications on the stability of ODNs.

In oocytes and embryos unmodified ODNs were degraded rapidly. In both cases, unmodified ODN was almost undetectable 10 min after injection. The rate of degradation of ODNs was significantly decreased by modification of several terminal internucleoside linkages. In embryos, for example, the half-life of an unmodified ODN was less than one minute while that of the modified derivative (An2-$N_3O_{10}N_2$) was approximately 15 min. The ODNs used as antisense inhibitors were probably even more stable, since they were modified with 2 additional phosphoramidates at the 3′ end of the sequence.

In both embryos and oocytes, exonucleolytic activity is the earliest detectable route of degradation of unmodified ODNs. By comparing the amount of intact 16-mer immediately following injection (Fig. 2A and 2B, lane 0), the embryonic exonuclease appears to be more active than that seen in oocytes. When the exonuclease is blocked by terminal phosphoramidate linkages, an endonucleolytic activity becomes apparent. In oocytes and embryos, both the pattern and the rate of degradation of the modified ODN are comparable.

Increasing the extent of chemical modification should further increase the stability of ODNs against embryonic endonucleases. Modified ODNs possessing fewer than eight consecutive phosphodiesters form heteroduplexes with RNA that can be degraded by E. coli and human RNase H activities (8,42,43), and may be more active in the Xenopus system than the

compounds used in the present study. We are currently investigating the stability and antisense activity of these more heavily modified analogs.

ODN-mediated mRNA degradation

Unmodified ODNs, although active in oocytes, do not function as effective antisense agents in Xenopus embryos, probably because they are more rapidly degraded. Sufficient amounts of an unmodified ODN can be injected into embryos to eliminate the target message, but such high levels of ODN cause non-specific toxic effects (15). The phosphoramidate analogs described in the present study are more stable, allowing far less ODN to be injected. The terminal phosphoramidate modifications provide complete exonuclease and partial endonuclease resistance, while the internal unmodified sequence allows the formation of heterduplexes with RNA that are substrates for RNase H. Phosphorothioates can be utilized in the central anionic region, but appear to be less active than the unmodified phosphodiesters.

Development of embryos injected with 2 ng of An2-NON was arrested shortly after gastrulation, presumably a consequence of reducing An2 mRNA levels by 80%. In contrast, normal embryogenesis occurred following injection of fertilized eggs with 5 ng of control modified ODNs. Injection of 0.2 ng of the anti-An2 ODN resulted in a 20% depletion of An2 mRNA, but did not appear to affect development. This may indicate that a threshold level of maternal An2 mRNA is required for normal embryogenesis. We are currently examining the level of An2 protein produced after injection of different concentrations of An2-NON to determine the effect on translation. Because An2 is thought to encode the α-subunit of mitochondrial ATPase, it will also be of interest to examine the effects of An2-NON on mitochondrial biogenesis.

Inhibition of cell division in vivo by anti-cyclin ODNs

Following the introduction of an anti-cyclin ODN into one half of a two cell embryo, cell division was inhibited in the blastomere receiving ODN. Embryos that received 10 ng of control ODN into one blastomere developed normally. This observation provides in vivo evidence for the importance of cyclin in cell division. In vitro antisense studies examining cyclin activity in Xenopus egg extracts suggest that cyclin B1 and cyclin B2 are functionally redundant since removing one or the other did not prevent entry into mitosis (39). Both cyclin B1 and B2 are components of Xenopus maturation-promoting factor and are present in the complex in roughly equivalent amounts (29). Utilizing modified ODNs specifically targeted against cyclin B1 or cyclin B2, it should now be possible to assay the relative importance of each protein for entry into mitosis.

The present studies establish that ODNs of the general structure phosphoramidate$_3$-phosphodiester$_8$-phosphoramidate$_4$ can be utilized to specifically degrade maternal messages in oocytes and embryos. The toxic effects seen previously with unmodified ODNs were eliminated by injecting only a few ng of the more active modified compounds. These new antisense inhibitors should prove generally useful in studies of early events of Xenopus embryogenesis.

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